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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Background: NKX3.1 is a homeoprotein with prostate-specific expression in adults. Loss of NKX3.1 correlates with prostate cancer progression. Loss of heterozygosity affects *NKX3.1* in about 80% of prostate cancers. This project focuses on DNA methylation of the *NKX3.1* gene promoter in prostate cancer cell lines and tissues.

Materials and methods: DNA was analyzed by methylation-specific PCR and sequencing of bisulfite-treated DNA. We also treated cultured cells with the methylation inhibitor 5-azacytidine and the histone deacetylase inhibitor, trichostatin A.

Results: There was no effect of 5-azacytidine or trichostatin A on *NKX3.1* expressionin cultured cells. By bisulfite modified DNA sequencing, we identified some methylated or partial methylated CpG islands in –1056 to 1172 of NKX3.1 gene. Some of them are selected to study their methylated situation in human prostate cancer tissues. We also study the effect of Sp transcription factor family on the expression of NKX.3.1, our results showed Sp1 and Sp3 did not influence on the expression of NKX3.1.

Conclusion: Some CpG islands in NKX3.1 gene are methylated or partial methylated.

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Introduction

The past year we have made substantial progress on the project. We have completed some important experiments in the gene methylation and expression of NKX3.1, a homeoprotein with prostate-specific expression in adults that might involve the prostate cancer development (1, 2). This annual report will first outline the data relevant to the specific aims in the grant. Here we also summarize our studies on the affect of SP1 transcription factor family on the expression of NKX3.1.

A. Analyze DNA methylation of CpG islands in the NKX3.1 5' upstream region and first exon HpaII/MspI analysis of prostate cancer cell line DNA. Use cell line DNA to develop a methylation-specific PCR assay for tissues. (Year 1)

1. HpaII/MspI digestion and Southern blotting of prostate cancer cell line DNA.

There are numerous CCGG sites located upstream and in the *NKX3.1* gene. Both *HpaII* and *MspI* can recognize CCGG site, but only *MspI* can cleave the sequence when the internal C residue is methylated. We isolated genomic DNA from prostate cancer cell lines by using Puregene DNA isolation Kit (Gentra Systems, Minneapolis, USA) with standard protocol. DNA (25μg) from different cell lines were digested with *HpaII* or *MspI* at 37 °C for 16 hours, then inactivate the enzymes by incubate at 65 °C 30 min. The digested products were run on 2% agarose gel over night, transferred to Zeta-Probe Blotting Membrane (BIO-RAD, Calif), hybridized with an *NKX3.1*-specific probe in Rapid-hyb buffer (Amersham life Science, NJ) according the standard protocol provided by the manufacturer. After hybridization, X-film was exposed on the membrane at –70 °C for 48 hours. The results showed that there were no differences in *HpaII/MspI* digestion patterns between LNCaP, DU-145 and PC-3 prostate cancer cell lines (data not shown), it may because that the CG islands in NKX3.1 are not fully methylated, but partially methylated as our data indicated below.

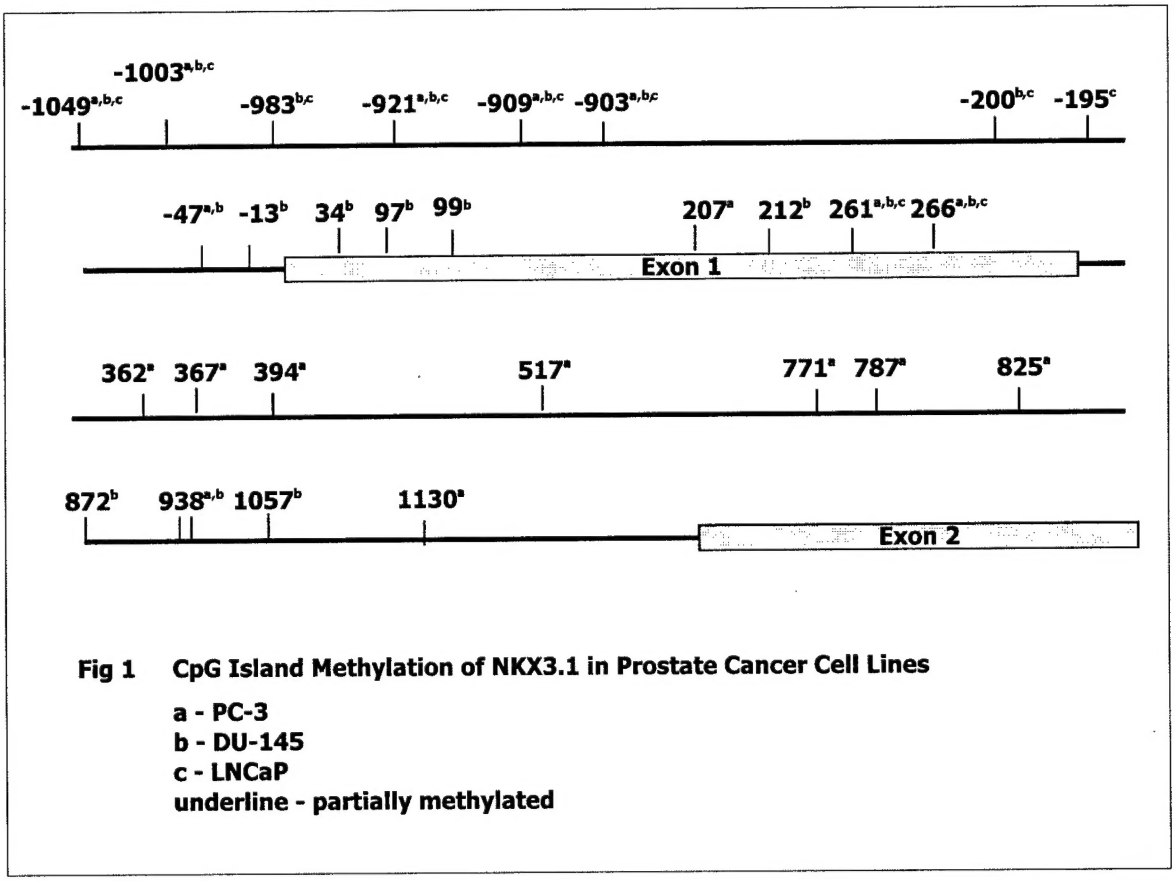

Fig 1 CpG Island Methylation of NKX3.1 in Prostate Cancer Cell Lines

a - PC-3
b - DU-145
c - LNCaP
underline - partially methylated

2. Synthesis of primers for methylation-specific PCR. Testing of primers on cell line DNA to compare NKX3.1 expressing and nonexpressing cell lines.

To determine primers for methylation-specific PCR, we used bisulfite-modified DNA sequencing method to look for the methylation status of NKX3.1. DNA (2μg) in a volume of 50μl was denatured in 0.3M NaOH at 42°C for 10min. Thirty μL of freshly prepared 10mM hydroquinone and 520μl of 3M sodium bisulfite (Sigma) at pH5.5 were added and mixed. The samples were covered with mineral oil and incubated at 50°C for 16hr. Modified DNA was desalted with the Wizard DNA clean-up system (Promega) according to the protocol provided by the manufacturer and resolved in 50μl sterile water. 5.5μl 3M NaOH was added into 50μl modified DNA solution and incubated at 37°C for 10min for denaturation, followed by ethanol precipitation with See DNA (Amersham Pharmacia Biotech) as coprecipitant. The DNA was resuspended in 20μl sterile water, stored at –20°C until use. Two μg bisulfite-modified DNA (about 100ng DNA before modification) were amplified by common or nested PCR with *NKX 3.1*-specific primers. Genomic sequencing of bisulfite-modified DNA was accomplished using purified PCR products as template. By this method, we identified partially methylated moieties in the *NKX3.1* gene. The results of methylated or partially methylated regions are summarized in Figure 1. The regions were focused from –1056 to 1172 in NKX 3.1 gene, including 5' frank region, exon Iand intron I. The CpG island methylation in this region was higher in PC-3 and DU-145 cells than in LNCaP cells, consistent with the expression profile of *NKX3.1* mRNA (1). Based on the data of bisulfite modified DNA sequencing, primers for methylation-specific PCR were determined and tested with prostate cancer cell lines and prostate tissues (see next part).

B. Examine promoter methylation in prostate cancer tissues with known expression levels of NKX3.1. Isolate nonmalignant and malignant prostate epithelial cells by laser capture microdissection (LCM) and analyze for NKX3.1 gene methylation by methylation-specific PCR. (Year 2)

1. Extraction of tissue DNA and analysis by methylation-specific PCR

After the block was marked for regions of prostate cancer and nonmalignant tissue, paraffin-embedded tissue was used to generate DNA with QIAamp DNA Mini Kit after removing paraffin wax by xylene extraction. DNA products were modified with bisulfite as above, then used for methylation-specific PCR with specific primers. Figure 2 shows the methylation-specific PCR results of two prostate blocks for the CG island at site –921 of NKX3.1. The primers for 1st PCR were sense: 5'- GGT ATT TTG AGA GGT TAA GGT AGG AGG ATT -3' and antisense: 5'- CCT ATT AAC TTT CCT TCC TCC CCA AAC ACA TA -3'; the primers for 2nd PCR are sense: 5'- GAG GTT GTA GTG AGT TAT GAT GGT -3' and antisense: 5'- CTA TAA CTA AAC TAA ACA ATA CCA TAA CAA CAA ACA -3 (for unmehtylation status) or 5'- CTA AAC TAA ACG ATA CCG TAA CAA CAA ACG -3' (for methylation status). PCR conditions for 1st PCR is 95 °C 15 min; 94 °C 1min, 52 °C 1 min, 72 °C 45s, 35 cycles; 72 °C 10 min, then keep at 4 °C. One twenty 1st PCR product was taken as template for 2nd PCR. PCR conditions for 2nd PCR is 95 °C 15 min; 94 °C 1min, 53 °C 45s, 72 °C 35s, 23-32 cycles; 72 °C 10 min, then keep at 4 °C. PCR products were run on 1.6% agrose gel in 1 x TBE buffer with EB (0.4 μg/ml). The target bands were visualized on UV light and take picture by Kodak digital science 1D. From these preliminary data, we can see that mathylation of the CpG island at position –921 of the *NKX3.1* gene was higher in cancer tissue compared with normal in the same patient. For this reason we will study this site in other cases

2. Laser capture microdissection of tissues to assess degree of methylation in cancers that either do or do not express NKX3.1

We are unsure that this element can be completed since we have found that about 90% of DNA is lost after bisulfite treatment (3). Therefore, we do not expect to be able to retrieve sufficient DNA from LCM for methylation-specific sequencing. We may be able to obtain sufficient DNA for methylation-specific PCR and we are addressing this with preliminary studies.

3. Analysis of samples for correlation with Gleason score.

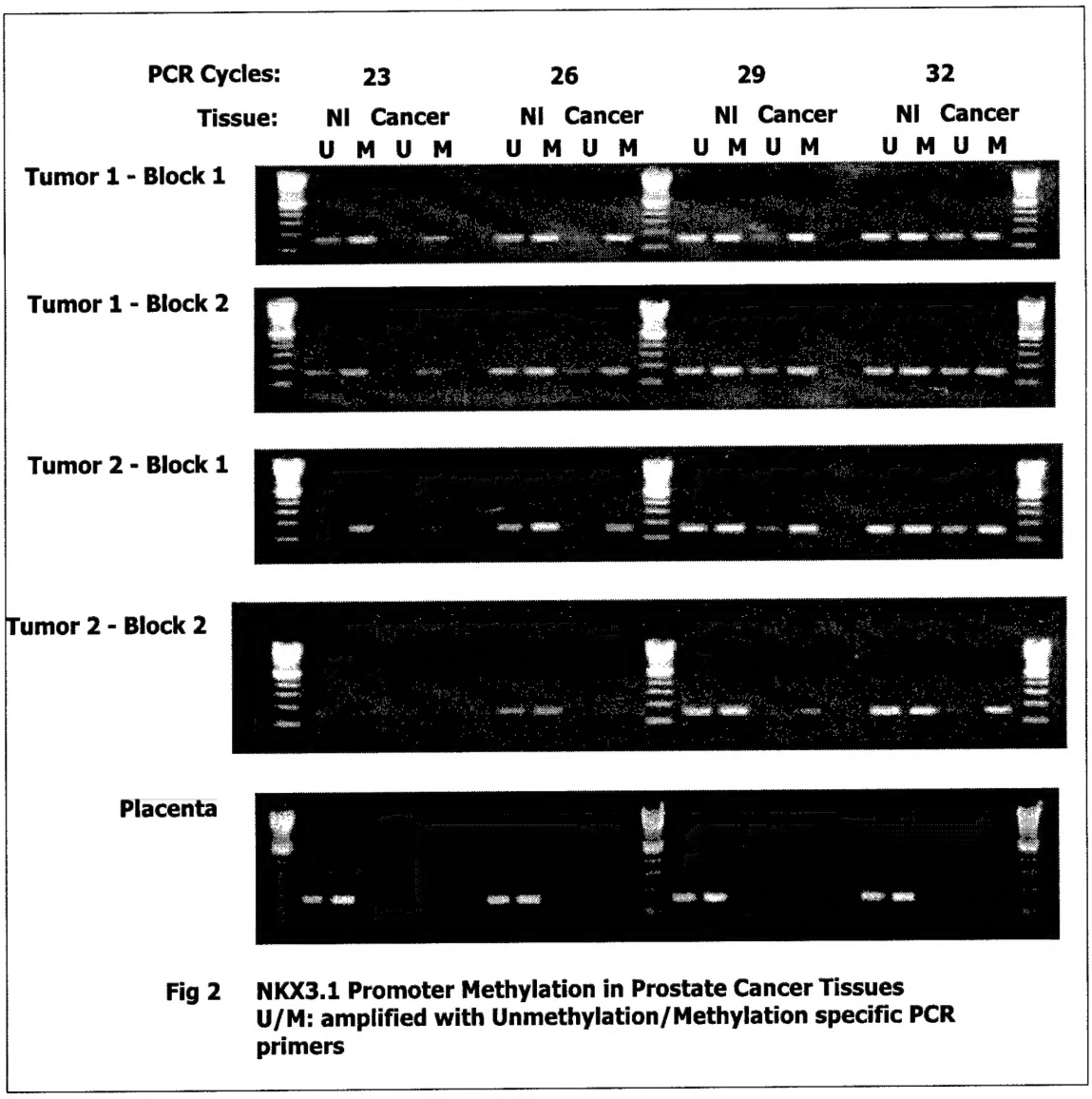

Fig 2 NKX3.1 Promoter Methylation in Prostate Cancer Tissues
U/M: amplified with Unmethylation/Methylation specific PCR primers

We have not as yet found CpG islands that are reproducibly methylated in prostate cancer allowing an analysis of correlation with Gleason grade. The feasibility of this element is still uncertain.

C. Activate expression of NKX3.1 in prostate cancer cell lines using chemical inhibitors of DNA methyltransferase (5-azacytidine) and histone deacetylase (trichostatin A). (Year 1-2)

1. Treatment of LNCaP, PC-3, DU-145 and TSU-Pr1 prostate cancer cell lines with 5-azacytidine or/and trichostatin A. *Analyze for NKX3.1 mRNA and protein expression. Analyze for change in methylation.*

The inhibitors of DNA methyltransferase (5-azacytidine) and histone deacetylase (trichostatin A) are commonly used to study the relationshop between DNA methylation and gene expression in cells (4). To determine whether control of NKX3.1 expression in cancer cell lines and tissues depended on gene methylation we treated cultured cells with methylation and histone deacetylase inhibitors and assayed *NKX3.1* mRNA expression by RT-PCR. We also analyzed protein expression by western blotting. Prostate cancer cell lines PC-3, DU-145 and LNCaP were cultured in modified IMEM (GIBCO) with 5% fetal bovine serum at 37°C with 5% CO_2 atmosphere. To test inactivation and reactivation, cells were seeded at 2×10^5 cells/T75 flask on day 0. After 24 hours, cells were treated with 5-aza-2'-deoxycytidine (5-AC) (Sigma) at a final concentration of 2μM, or with trichostatin A (TSA) (Wako) at a final concentration 200ng/ml, or with both 5-AC and TSA for 24 to 72 hours, exchanging the medium every 24 hours. Identical volumes of solvent alone were used as controls. Total RNA was extracted by RNeasy Mini Kit (Qiagen Inc.Valencia, CA) and treated with DNase (Qiagen Inc.Valencia, CA) to limit the DNA contamination. The purified RNA was

stored at –20°C until use.

RT-PCR primer sequence for NKX3.1 were sense 5'agc cag agc cag agc cag agg 3', antisense: 5' ttg ggt ctc cgt gag ctt gag gtt 3'; RT-PCR product is 332 bp. RT-PCR reaction was done with One-step RT-PCR Kit (Qiagen Inc.Valencia, CA). The RT-PCR conditions are as follows: 50°C 30min for reverse transcription, 95°C 15min for initial PCR activation step, then 95°C 1 min, 55°C 1min, 72°C 1min for 30 cycles. RT-PCR products were run on 1.6% agrose gel in 1xTBE buffer with EB (0.4 μg/ml). The target bands were visualized on UV light and take picture by Kodak digital science 1D. Figure 3 and figure 4 shows the results of these experiments. We also found no effects of 5-AC and TSA on NKX3.1 protein expression by western blotting in the three cell lines (data not shown).

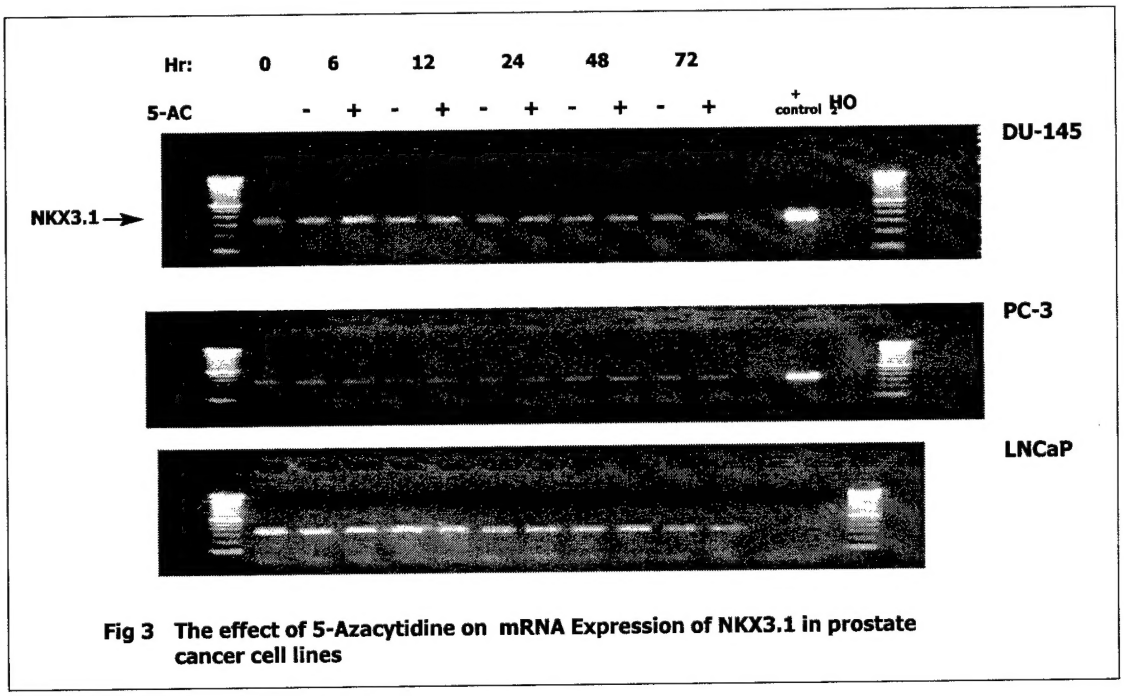

Fig 3 The effect of 5-Azacytidine on mRNA Expression of NKX3.1 in prostate cancer cell lines

To be sure that the 5-AC and TSA were effective, we analyzed expression of androgen receptor (AR) in DU-145 cells after treatment with these agents. Figure 5 shows that consistent with published reports (5) we were able to activate *AR* expression in DU-145 cells. Based on these results, there was no effect of 5-AC and TSA on *NKX3.1* expression in these prostate cancer cell lines. The analysis of *NKX3.1* methylation status in prostate cancer cell lines after treatment by inhibitors of methylation and histone deacetylase was not addressed.

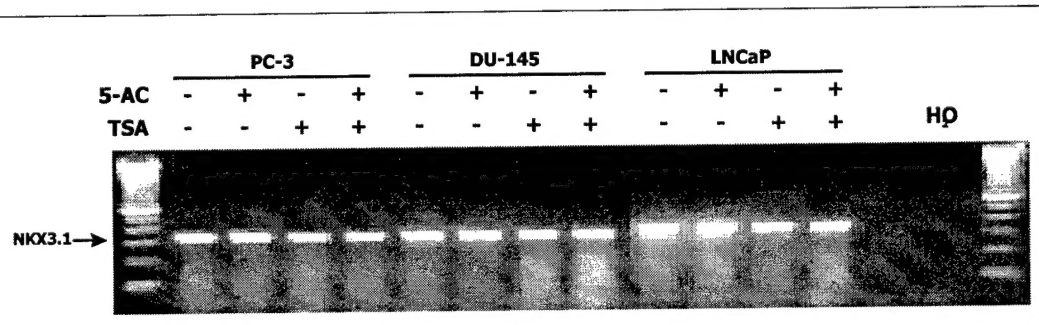

Fig 4 The effect of 5-Azacytidine and Trichostatin A on mRNA Expression of NKX3.1in prostate cancer cell lines. Culture time was 24 hours

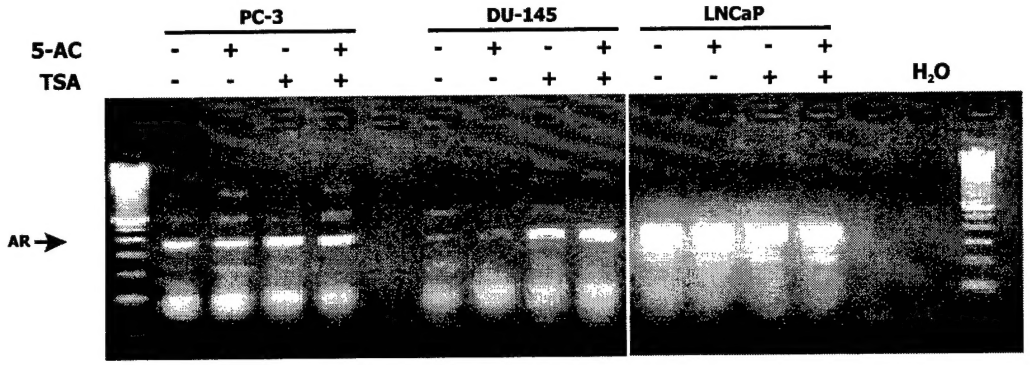

Fig 5 The effect of 5-Azacytidine and Trichostatin A on Androgen Receptor mRNA Expression

D. Sp transcription factor and expression of NKX.3.1

Sp transcription factor family contains at least four members that regulate transcription of a large number of genes (6). There are five GGGCGG Sp1 binding sites in the *NKX3.1* upstream region 3' to –1200, -1003; -298; -147; -104; -36. It is possible that the Sp transcription factors may play a role in the regulation of NKX3.1 expression. Since the Sp1 binding sites contain a CpG site, the methylation status of CpG would influence on Sp1 binding to DNA. We have begun to study the possible role of Sp1 and Sp3 in the regulation of NKX3.1 expression.

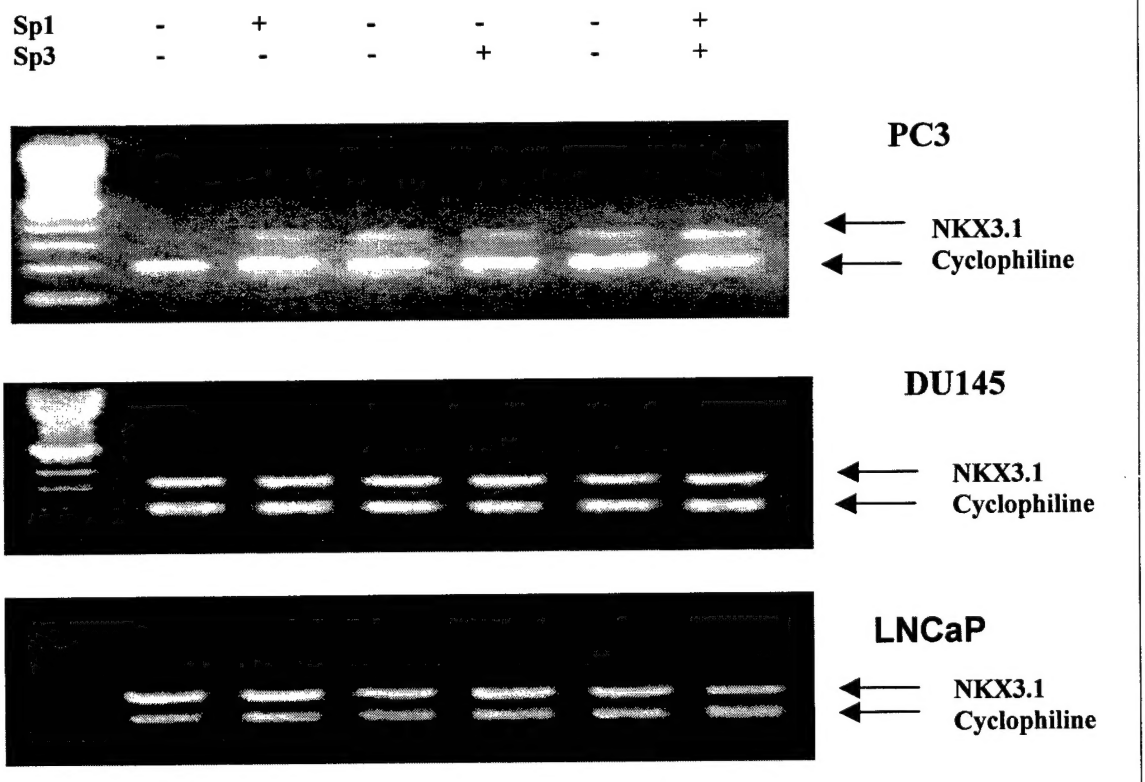

Figure 6 mRNA level of NKX3.1 in prostate cancer cell lines after infection with Sp1 and/or Sp3

We used Sp1 and/or Sp3 transcription vectors infected prostate cancer cell lines. After 48 hours culture, the infected cells were harvest and then extracted RNA by Qiagen kit as described above. The NKX3.1 expression was detected by RT-PCR with One-step RT-PCR Kit (QIAgene). A housekeeping gene, cyclophilin, was coamplified with *NKX3.1* as control. The primer sequences of cyclophilin are sense 5'atg gtc aac ccc acc gtg ttc ttc g 3', antisense: 5'cgt gtg aag tca cca ccc tga cac a 3', the RT-PCR product of cyclophilin is 206bp. The primer ratio is 5:1 (NKX3.1:Cyclophilin). RT-PCR conditions are as described above, except the numbers of PCR cycles ranged from 26 to 35.

Our data showed that the mRNA level of NKX3.1 in all three prostate cancer cell lines showed no significant change after transfection of Sp1 and /or Sp3 vectors (Figure 6). The protein level of NKX3.1 in PC3 is similar with the mRNA data (no change) after transfection with Sp1 or Sp3 (data not show).

Summary

Based on our current data, we can make several conclusions as follows:

1. Inhibitors of methylation and histone deacetylase, 5-AC and TSA, have no effect on *NKX3.1* expression in prostate cancer cell lines.
2. Some CpG islands located in –1056 to 1172 of NKX 3.1 gene showed methylated or partially methylated. Their relationship with NKX3.1 expression is valuable for further study.
3. The role of Sp transcription factor family in the regulation of NKX3.1 expression is needed to further study.

Work for next step

For the further study of relationship between CpG island methylation and NKX3.1 expression, some experiments will be done.

1. We will test more methylation sites CpG islands at NKX3.1 gene and determine methylation status of NKX3.1 gene with more prostate cancer specimens. All the works will be finished at the end of this year.
2. We will analyze for methylation after treatment prostate cancer cell lines by inhibitors of methylation and histone deacetylase
3. We will use point mutation technology to change the CpG site within Sp binding sites in the vector inserted promoter of NKX3.1, then co-infect cells with these mutant vectors and Sp1 and Sp3, to see whether Sp1 and Sp3 play a role in NKX3.1 expression or not.

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